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(57) Abstract

The invention provides a composition comprising physiologically tolerable vesicles which comprise (i) a vesicle membrane forming material, (ii) at least one chromophore material having a light absorption maximum in the range 300 to 1300 nm, and (iii) a scavenger, optionally together with at least one pharmaceutically acceptable carrier or excipient.

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Methods and compositions for medical imaging

The present invention relates to vesicular compositions, in particular vesicular light imaging contrast media, and to their diagnostic use.

Light imaging is a diagnostic imaging modality in which light passing through, reflected from or generated in an object is used to provide spatial or functional information about the object, information that can generally be presented in the form of an image. In the clinical setting, light imaging can reveal the presence of abnormal structures, e.g. lesions, in organs such as the skin, breast and prostate. By elucidating the chemical and physical properties of such lesions light imaging can assist in classifying them as benign or malignant.

Light passing through body tissue is both scattered and absorbed. Absorption will occur where the tissue contains a chromophore which absorbs light of the incident wavelength. Some natural biomolecules, such as hæmoglobin, strongly absorb visible light. The absorption of visible light is strongest for wavelengths below 600 nm thus giving body tissue its characteristic colour and setting a practical lower limit on the wavelengths of light that can be used for imaging or therapy.

Imaging and therapy with light having a wavelength between 300 and 650 nm is practical only on a body surface or just beneath such a surface. Examples of surfaces that can be imaged are the top layer of the skin, surfaces that can be reached by an endoscope, laparoscope or other imaging device, and surfaces that

are surgically exposed.

A practical upper limit for the useful wavelengths of light to be used for internal imaging of the body is set by the absorbing properties of water and fats in the body. Accordingly, the preferred "window" for internal imaging and therapy extends from about 650 to 1100 nm, i.e. in the near infrared.

In normal tissue the absorption coefficient for light in this wavelength range is 0.1 to 10 cm^{-1}: effectively an average photon will travel for 0.1 to 10 cm before being absorbed. However the scattering coefficient for infrared light in normal tissue is typically 10^1 to 10^3 cm^{-1}, meaning that the average photon travels only 10^{-3} to 10^{-1} cm before suffering a scattering event. Light scattering in body tissue is Mie-like and thus incident light does initially retain a relatively higher intensity in the direction of incidence. Nevertheless, direction scrambling is essentially complete after a fraction of a centimetre, e.g. after about 0.2 cm for light of wavelength 950 nm passing through human blood. Scattering is a much more serious impediment to light imaging in the available wavelength window than is absorption. Besides altering the direction of propagation of each photon, scattering extends the length of the overall path each photon follows through the body, thereby increasing the probability that the photon will be internally absorbed.

Even with the significant absorption and scattering of light that occurs in body tissue, it is still feasible to generate usable light images; indeed, some scattering and/or absorption is necessary if contrast between different organs or tissues is to be obtained in light images.

Naturally occurring substances may assist in image contrast generation if they are distributed non-uniformly among adjacent organs or organ components. However, it is especially preferred to administer contrast agents which will enhance contrast in the light image. Such administered contrast agents may be materials which scatter, absorb and/or emit light.

Scattering agents will generally be particulate materials with particle sizes comparable to the wavelength of the incident imaging light. Such particles may for example be solid particles, hollow spheres with intact or perforated walls, liquid droplets, or vesicles containing gases or liquids. Suitable scattering agents are described in more detail in our international patent publication No. WO96/23524, the entire contents of which are incorporated herein by reference.

Absorbing and emitting agents will comprise inorganic or organic dyes that absorb, fluoresce or phosphoresce, i.e. materials which contain a "chromophore", or substances that generate light through chemiluminescence or bioluminescence.

A dye compound may form the radiation absorbing component or may be a part thereof. A dye compound or composition is a substance that absorbs electromagnetic radiation with a wavelength of 300 nm to 1300 nm, generally with a change in the populations of the electronic energy levels of the substance.

By "chromophore" is meant a group in a composition of matter (e.g. an organic or inorganic group) which absorbs and/or emits light. The term thus includes fluorophores, groups which are fluorescent, as well as phosphorescent groups. In general, chromophores will

contain a complexed metal ion or an extensive delocalized electron system. The present invention is particularly concerned with the latter type, i.e. with compounds having an optionally metallated organic chromphore. The term chromophore may be used herein to designate a chromophore group or a compound containing such a group.

Such dyes or substances may be in solution or alternatively may be present in a particulate agent (e.g. solid particulates), emulsion droplets or gas, solid or liquid containing vesicles (e.g. micelles, liposomes, microbubbles, microballoons, etc.). Such particulate agents may accordingly function both as light scatterers and as light absorbers/emitters.

The present invention is concerned in particular with chromophore-containing vesicular agents. Such agents are described for example in WO96/23524.

Chromophore-containing agents function by absorption of light photons by the chromophore which boosts the electronic state of the chromophore from the ground state to an excited state. For a dye molecule in solution, the excited molecule initially produced on photoabsorption will generally be unfavourably solvated and will be strained. Through solvent rearrangement and changes in conformation the molecule drops to an energy minimum for the excited state (generally, like the ground state, a singlet state) within a few molecular vibrations with the release of heat. Thereafter the molecule may return to its ground state through a non-radiative process with loss of energy to the surroundings - this is referred to as internal conversion or non-radiative decay. Alternatively it may fluoresce, returning to ground state with the emission of a photon. Because part of the energy initially

contained in the absorbed photon is converted into heat, the emitted photon generally has lower energy and longer wavelength than the absorbed photon.

Where non-radiative and fluorescence decay are inefficient, the excited singlet state can convert through intersystem crossing to an excited state of different total spin quantum number (generally a triplet state). This second excited state can decay back to the ground singlet state with photon emission as phosphorescence; however, since the transition is symmetry forbidden, photon emission as phosphorescence occurs with a much larger time delay than does photoemission as fluorescence. Moreover, since the second excited state is long lived, it is available for chemical reaction.

One important chemical reaction of the second excited state that can occur is the production of singlet oxygen. The ground state of oxygen differs from that of most organic molecules in being a triplet state; however, through interaction with the excited triplet state of a dye, oxygen may be transferred to the excited singlet state with simultaneous relaxation of an excited triplet state dye into its ground singlet state. Singlet oxygen is a highly reactive species that can attack many organic molecules, especially those with extended unsaturated bond systems. Most organic dyes contain extended unsaturated bond systems and thus carry the keys to their own degradation. They promote singlet oxygen formation and are also highly susceptible to its destructive action.

It is not only the dye itself that is susceptible to photodecomposition as a result of irradiation of the dye. In general any molecule in the vicinity of an irradiated dye may be attacked by singlet oxygen

produced by the dye's excited state. Indeed, photodynamic therapy actively uses this effect to selectively kill cancer cells by irradiating photosensitive dyes accumulated at the tumor site.

Even in the absence of singlet oxygen, photochemical degradation of dye molecules may occur, e.g. by hydrogen abstraction from neighbouring molecules by the excited triplet state of the dye. Radicals thus formed may then undergo further reaction and decomposition. They may also react with chemical components of the body.

Accordingly, where a chromophore is to be used as a light imaging contrast agent it carries some risk of undesired phototoxicity.

The present invention is directed to the provision of chromophore-containing vesicular light imaging contrast agents with enhanced stability and reduced risk of phototoxicity.

Viewed from one aspect therefore the present invention provides a vesicular composition comprising physiologically tolerable vesicles comprising (i) a vesicle membrane forming material, (ii) at least one chromophore-containing material (for example free or attached to a lipophilic moiety which may be capable of participating in vesicle membrane formation) having a light absorption maximum in the range 300 to 1300 nm, and (iii) a scavenger for reactive species (e.g. a radical scavenger, an antioxidant, singlet oxygen quencher, chain transfer agent or any of the materials discussed below), optionally together with at least one pharmaceutically acceptable carrier or excipient.

In the compositions of the invention, the chromophore material and the scavenger may be dispersed within the

central void of the vesicle, e.g. within the fluid core of a liposome, or alternatively either or both of the chromophore material and the scavenger may be associated with the vesicle membrane, e.g. being bonded to or laminated to or pendant from the exterior or interior surfaces of the membrane or held wholly or partially within the membrane itself, or forming at least part of the vesicle membrane.

Where the chromophore or scavenger materials are associated with the membrane, this may for example be as a result of their attachment, e.g. non-covalent attachment (such as adsorbed onto or absorbed into the membrane or intercalated within the membrane) or covalent attachment, to a membrane forming molecule, or to a hydrophobic (or less commonly hydrophilic) "anchor" group that penetrates the membrane while leaving the rest of the chromophore or scavenger pendant from the membrane. Alternatively, the scavenger molecules may constitute at least part of the membrane-forming material (i.e. the phospholipid), especially when that material is subject to attack by singlet oxygen, free radicals, or other reactive species. Additionally, for example, the scavenger molecules may reside at the interface between the aqueous phase and the liposomal membrane in a laminar fashion. Alternatively, scavenging molecules as a whole may constitute the membrane forming material.

Similarly, chromophore materials can be modified so that as a result of their attachment (e.g. covalent attachment) to a group or substance, the chromophore will become disposed on the surface of an aggregate (e.g. a vesicle) or a biological molecule or structure (e.g. an indigenous protein or lipoproteins). Examples of such groups and materials include single alkyl groups, hydrophobic groups, membrane forming materials

which associate with micelle forming materials (e.g. Pluronics, Tetronics, Tweens, Spans, and other physiologically tolerable surfactants) as well as novel, polymeric micelle forming materials. Thus, in a formulation of different sized particles, one set of particles could be chromophore-containing micelles with or without attachments and the other set could be larger liposomal particles. The molecules in the micelles would then be expected to partition rapidly out of the micelles and onto naturally resident proteins and lipoproteins, such as albumin, and thereby target leaky vascular beds such as tumors and sites of inflammation, while the larger, liposomal chromophore would be expected to circulate within the vascular bed.

In addition, chromophores with attachments that do not form membranes but do add lipophilicity to the molecule could be formulated in vesicle preparations (e.g. micelles, liquid crystals, vesicles, mixed solvents, etc.) such that upon injection, they adhere to normally resident proteins and lipoproteins for transport to vascular targets and lymphatic targets for intraoperative use, as is known to occur for albumin molecules modified with blue dyes for intraoperative advantage. Thus, at low levels, the modified chromophores would adhere to the blood resident albumin and thereby circulate and percolate into sites of leaky vasculature. The resulting albumin/chromophore structures thereby become the smaller of the mixed particles for injection.

Desirably, the distribution of the scavenger in or on the vesicles should be similar to that of the chromophore - i.e. if the chromophore is disposed about the external surface then the scavenger should also be disposed in this fashion. Where the chromophore is disposed in the vesicle membrane or within the vesicle

core (e.g. in solution in the core space or pendant from the interior membrane surface) then it may be sufficient for the scavenger to be pendant from the interior membrane surface or in solution in the core space. If desired, further scavenger may be present in any liquid medium in which the vesicles are dispersed.

Since each chromophore may cause the generation of a plurality of destructive species such as singlet oxygen, the vesicles preferably contain the scavenger in a mole ratio of at least 1:1 relative to chromophores, preferably at least 2:1, especially 3:1 to 10:1. The vesicles of the present invention are preferably unilamellar or multilamellar liposomes but may if desired have other vesicular structures, e.g. micelles, microballoons, microsponges, etc. Optionally, the surfaces of the vesicles may be coated with a blood residence time prolonging agent, e.g. a polyalkylene oxide such as a polyethylene glycol or a heparinoid or other synthetic or natural substance.

Examples of suitable scavengers include mannitol, salicyclic acid, trolox esters, tryptophan, cysteamine, superoxide dismutase, catalase, their polyethylene oxide conjugates and any other material highly reactive with singlet oxygen or other radiation products. Further examples include persistent free radicals, such as the trityl radicals disclosed in WO 91/12024. Such scavengers may be singlet oxygen scavengers, singlet oxygen quenchers, radical traps or peroxide traps. Water-soluble such substances may be disposed in aqueous solution in a liposome core. Alternatively, such materials may be anchored to vesicle membranes by attachment of lipophilic tails, e.g. an alkylene, alkenylene, alkynylene or aralkyl tail, generally having a carbon content of 10 to 30 carbons, preferably 15 to 25 carbons. The length of such tails is preferably

comparable to the tail length of the membrane forming lipid.

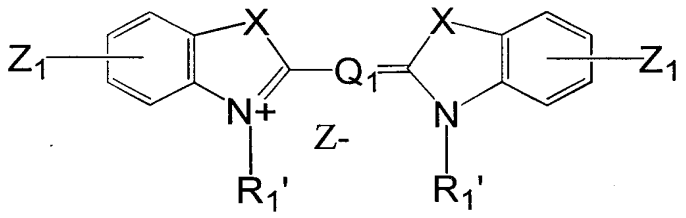
To further emphasize their amphiphilic nature and promote their incorporation into the membrane, the scavengers may be modified to include hydrophilic groups such as hydroxyalkyl or, more preferably, oxyacid groups, e.g. sulphate groups.

Alternatively, a scavenger molecule can be conjugated to (i.e. reacted to bond with) a membrane forming compound, e.g. a phospholipid. Alternatively, the scavenger molecule may constitute the membrane-forming material (i.e. the phospholipid). In this case, the specific addition of scavenging species to the liposomes may be unnecessary.

The chromophores may likewise be functionalized to include lipophilic (hydrophobic) tails or hydrophilic moieties or may be conjugated to membrane forming materials.

The chromophores in the vesicles of the invention may be of any convenient dye class, e.g. phthalocyanines, naphthalocyanines, cyanines, squaryliums, pyryliums, thiapyryliums, anthraquinones, naphthaloquinones, triphenylmethanes, azo dyes, merocyanines, perylenes, and metal-complexed dyes (such as porphyrins), or a mixture of these dye classes (if desired, within the same vesicle). In general any dye absorbing in the desired wavelength range (300-1300 nm) may be used. For maximum incorporation into vesicles, the dye will preferably have high solubility in organic solvents and low solubility in water or should be amphiphilic.

Typical examples of appropriate chromophores include the compounds of formula I



(I)

wherein

each Z_1 is independently selected from the group consisting of:

hydrogen,

a methyl group optionally substituted with a substituent selected from the group consisting of hydroxyl, carboxyl, sulfonate, phosphonate, and poly(alkylene oxidyl) such as ω-hydroxypoly(ethylene oxidyl) and ω-methoxypoly(ethylene oxidyl) the molecular weights of which can be up to about 50,000,

an ethyl group optionally substituted with one or two substituents selected from the group consisting of hydroxyl, carboxyl, sulfonate, phosphonate, and poly(alkylene oxidyl) such as ω-hydroxypoly(ethylene oxidyl) and ω-methoxypoly(ethylene oxidyl) the molecular weights of which can be up to about 50,000,

an ethylene group optionally substituted with one or two substituents selected from the group consisting of hydroxyl, carboxyl, sulfonate, phosphonate, and poly(alkylene oxidyl) such as ω-hydroxypoly(ethylene oxidyl) and ω-methoxypoly(ethylene oxidyl) the molecular weights of which can be up to about 50,000,

a C_{3-16} alkyl group, the alkyl portion of which is optionally substituted as defined above,

a C_{1-16} alkoxyl ether group, the alkyl portion of which is optionally substituted as defined above,

a C_{1-16} carboxyalkyl ester group, the alkyl portion of which is optionally substituted as defined above,

a C_{1-16} oxycarbonylalkyl ester group, the alkyl portion of which is optionally substituted as defined

above,

a C_{1-16} carbonylaminoalkyl amide group, the alkyl portion of which is optionally substituted as defined above,

a C_{1-16} aminocarbonylalkyl amide group, the alkyl portion of which is optionally substituted as defined above,

a carboxylic acid group which may be a carboxylate group,

a sulfonate group,

a hydroxyl group,

a phosphate group,

a C_{1-16} sulfonamidoalkyl group, the alkyl portion of which is optionally substituted as defined above,

a C_{1-16} aminosulfonylalkyl group, the alkyl portion of which is optionally substituted as defined above,

a C_{1-16} aminocarbonylaminoalkyl urea group, the alkyl portion of which is optionally substituted as defined above,

a C_{1-16} aminothiocarbonylaminoalkyl thiourea group, the alkyl portion of which is optionally substituted as defined above,

a phenyl-C_{1-16}-alkyl group, the alkyl portion of which is optionally substituted as defined above,

a phenoxy-C_{1-16}-alkyl group, the alkyl portion of which is optionally substituted as defined above,

a C_{1-16} phenyloxyalkyl group, the alkyl portion of which is as defined above,

an oxyphenoxy-C_{1-16}-alkyl group, the alkyl portion of which is optionally substituted as defined above,

a poly(alkylene oxidyl) group such as hydroxypoly(ethylene oxidyl) and methoxypoly(ethylene oxidyl) with a molecular weight up to about 50,000,

and an annulated aromatic ring which comprises a benz[e]aromatic ring, a benz[f]aromatic ring, or a benz[g]aromatic ring, where e, f, and g are defined relative to the indole structure as a template and each

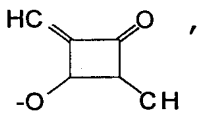
of which ring may be substituted by C_{1-16} alkyl, C_{1-16} alkoxyl, carboxyl, sulfonate, sulfonamido, phenyl, poly(alkylene oxidyl) or phenoxyl groups as defined above;

each R_1' is independently selected from the group consisting of methyl and a C_{2-16} alkyl group including a substituted alkyl group where alkyl is optionally substituted as defined above,

each X is independently selected from the group consisting of O, N-R_1', S, Se, Te, CH=CH, and $(CH_3)_2C$; and

Q_1 is selected from the group consisting of:

$(CH=CH)_n$ where n has a value of 1 to 6,



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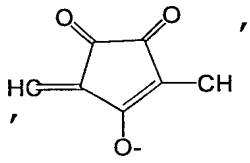

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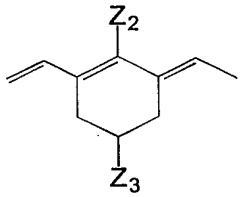
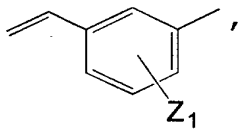


where Z_2 is selected from the group consisting of H, chloro, O-alkyl, S-alkyl, where alkyl is optionally substituted as defined above, O-poly(alkylene oxidyl), S-poly(alkylene oxidyl), where poly(alkylene oxidyl) is as defined above and also includes poly(alkylene oxide) groups to which another dye is attached at the ω-end, O-phenyl, S-phenyl, wherein the phenyl groups may be substituted with alkyl groups optionally substituted as defined above, O-alkyl groups as defined above, S-alkyl groups as defined above, aminothiocarbonylaminoalkyl

groups, and aminothiocarbonylaminophenyl groups, and

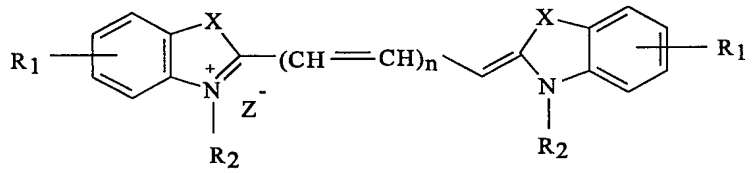
where Z_3 is selected from the group consisting of H, carboxylate, and carboxyalkyl where alkyl is as defined above, carbonylaminoalkyl where alkyl is as defined above, and carbonylaminophenyl where phenyl is as defined above, and



and

Z^- is a physiologically tolerable counterion, preferably I, Br, Cl, or OAc.

More preferably, dyes of formula II may be used:



(II)

wherein

n is an integer having a value of 1 to 6;

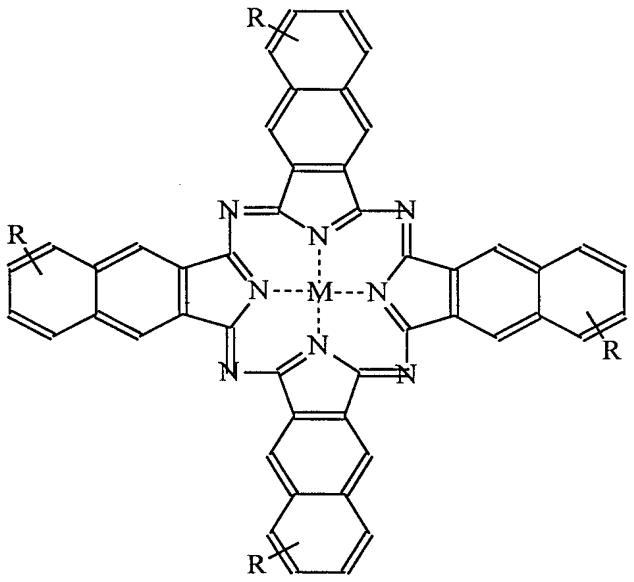
each R_1, which may be the same or different, represents a hydrogen atom or a solubilizing group, or adjacent R_1 substituents together with the ring carbons to which they are attached may form a ring structure, preferably a 5- or 6-membered ring, for example an aromatic ring;

each R_2, which may be the same or different, represents a hydrogen atom or a lipophilic group, e.g. an optionally unsaturated C_{1-24} alkyl group, preferably a C_{6-18} group, especially when the dye is to be used as part of a gas-containing non-solid particle, alternatively, each R_2 may be an optionally unsaturated C_{2-8} alkyl group which is attached to one or more solubilizing groups;

each X, which may be the same or different, each represents O, S, -CH=CH- or $C(R_3)_2$ in which each R_3, which may be the same or different, represents a hydrogen atom or more preferably a methyl or ethyl group;

Z is a physiologically tolerable counterion, preferably I, Br or Cl.

Other examples of appropriate chromophores are the compounds of formula III



(III)

(where each R is independently selected from C_{10-30} alkyl, alkenyl, alkynyl, arakyl and alkyl-aralkyl groups and M is a physiologically tolerable metal ion or polyatomic complex (e.g. a metal oxide or sulphide with one or more metal atoms and one or more oxygen and/or sulphur atoms)).

Such lipophilic dyes with side chains are known from the literature and may be prepared by conventional organic synthetic techniques.

Further examples of appropriate chromophores may be found in "Topics in Applied Chemistry: Infrared Absorbing Dyes", Ed. Matsuoka, Plenum, NY, 1990.

Any physiologically tolerable vesicle structure may be used in the compositions of the invention. Preferred however are liposomes.

A liposome is conventionally defined as a particle comprising a bilayer of amphiphilic molecules arranged such that the hydrophobic portions of the molecules are packed together within the bilayer while the hydrophilic parts are exposed to the aqueous solution either outside the particle or present in the interior space of the particle. Such molecules can be either naturally occurring amphiphiles (i.e. phospholipids) or synthetic molecules with the single restriction that they must have a double tailed hydrophobic portion. For naturally occurring amphiphiles, the "tails" are composed of long chain fatty acids either saturated or containing one or more double bonds. Synthetic amphiphiles are often based upon an amine (i.e. cationic) or carbon linkage between the double hydrophobic groups and the hydrophilic "head" groups. Novel liposomes have been reported which form spontaneously from anionic and cationic single tailed surfactants acting in concert to form bilayer structures.

Preferred groups of liposomes are phospholipid liposomes and multilamellar liposomes. Also suitable are phospholipid liposomes containing cholesterol derivatives (US-A-4544545); liposomes associated with compounds containing aldehydes (US-A-4590060); lipid matrix carriers (US-A-4610868); liposomes containing triiodobenzoic acid derivatives of the type also suitable for X-ray examination of liver and spleen (DE-A-2935195); X-ray contrast liposomes of the type also

suitable for lymphography (US-A-4192859); receptor-targeted liposomes (WO-87/07150); immunoactive liposomes (EP-A-307175); liposomes containing antitumor antibodies (US-A-4865835); liposomes containing macromolecular bound paramagnetic ions of the type also suitable for MRI (GB-A-2193095); phospholipid liposomes of the type also suitable for ultrasound imaging containing sodium bicarbonate or aminomalonate as gas precursor (US-A-4900540); stable plurilamellar vesicles (US-A-4522803); oil-filled paucilamellar liposomes containing non-ionic surfactants as the lipid (US-A-4911928); liposomal phospholipid polymers containing ligands for reversible binding with oxygen (US-A-4675310); large unilamellar liposomes containing non-ionic surfactants (US-A-4853228); aerosol formulations containing liposomes (US-A-4938947 and US-A-5017359); liposomes containing amphipathic compounds (EP-A-361894); liposomes produced by adding an aqueous phase to an organic lipid solution followed by evaporating the solvent and then adding aqueous lipid phase to the concentrate (FR-A-2561101); stable monophasic lipid vesicles of the type also useful for encapsulation of bioactive agents at high concentrations (WO-85/00751); homogeneous liposome compounds comprising suspensions in liquefiable gel (US-A-5008109); lipospheres (solid hydrophilic cores coated with phospholipid) of the type also suitable for controlled extended release of active compounds (WO-91/07171); liposomes sequestered in gel (US-A-4708861); metal chelates bound to liposomes, also suitable for use as MR contrast agents (WO-91/14178); lipid complexes of X-ray contrast agents (WO-89/11272); liposomes that can capture high solute-to-lipid ratios (WO-91/10422); liposomes containing covalently bound PEG moieties on the external surface to improve serum half-life (WO-90/04384); contrast agents comprising liposomes of specified diameter encapsulating paramagnetic and/or superparamagnetic agents (WO-90/04943); liposomes of the

type also suitable for delivering imaging agents to tumors consisting of small liposomes prepared from pure phospholipids (EP-A-179444); encapsulated X-ray contrast agents such as iopramide in liposomes (US-A-5110475); non-phospholipid liposome compositions (US-A-5043165); hepatocyte-directed vesicle delivery systems (US-A-4603044); gas-filled liposomes of the type also suitable as ultrasound contrast agents for imaging organs (US-A-5088499); injectable microbubble suspensions stabilized by liposomes (WO-91/15244); paramagnetic chelates bound to liposomes (US-A-5135737); liposome compositions of the type also suitable for localizing compounds in solid tumors (WO-91/05546); injectable X-ray opacifying liposome compositions (WO-88/09165); liposomes encapsulating iron chelates (EP-A-494616); liposomes linked to targeting molecules through disulfide bonds (WO-90/07924); compositions consisting of non-radioactive crystalline x-ray contrast agents and polymeric surface modifiers with reduced particle size (EP-A-48482). Alternatively, liposomes can be spontaneously formed after combining single tailed cation and ionic surfactants (E.W. Kaler *et al*, Phase Behavior of Aqueous Mixtures of Anionic and Cationic Surfactants Along a Dilution Path, in *Structures and Dynamics of Strongly Interacting Colloids and Supramolecular Aggregates in Solution*, 1992, Kluwer Academic Publishers, pages 571-577).

Liposomes and other vesicles may be prepared in conventional fashion from the membrane-forming materials, chromophores and scavengers and any other optional components, e.g. by extrusion of an aqueous mixture containing these components, by coacervation, or by sonication of an organic/aqueous two-phase mixture containing water soluble membrane forming materials and stabilizers and chromophores which are less soluble in the water phase than the organic phase, followed if

required by removal of the organic solvent.

Preferably the vesicle size is in the range 0.02 to 20 μm, especially 0.05 to 12 μm, more especially 0.1 to 0.8 μm. Such sizes can be achieved by particle separation or extrusion techniques or by use of a microfluidizer.

For optimum utility the vesicles should be tailored to have an average size that favourably controls their biodistribution and clearance. In one embodiment of the invention vesicles of two characteristic sizes may be present, each containing a scavenger and a different chromophore. In general, the different vesicles will be prepared in separate aqueous suspensions which may be mixed prior to administration or may be administered separately.

Cancerous tumors generally have more highly developed vasculature than normal tissue. However, the blood vessels usually are "leaky", and dyes dissolved in the blood passing through the tumor tend to accumulate in the extracellular fluid as they leak out of the blood vessels. When contrast agents containing vesicles of two different sizes are administered, the smaller vesicles will tend to leak from the blood vessels, while the larger vesicles will tend to be retained.

With such compositions, tumors may therefore be detected as a result of the different rates of clearance of the vesicles from the tumor site. This can be followed using irradiation with light at the different characteristic frequencies of the different chromophores. The time dependance of the signals from the two types of vesicle will provide comprehensive characterization of the tumor vasculature.

This use of two (or more) differently sized, distinctively labelled particles, is novel and forms a further aspect of the invention. In this aspect, the particles need not be vesicles, although they preferably are, and they need not contain chromophores or scavengers, although they preferably do. Viewed from this aspect the invention provides a method of detecting tumors in a human or animal body, said method comprising parenterally administering to said body, sequentially, separately or preferably simultaneously, at least two populations of physiologically tolerable particles, each said population being labelled with a material detectable in the same diagnostic imaging modality, the mean particle size being different for each population and the labelling material being different for each population and interdistinguishable in said imaging modality, the particle size for one population being such that said particles pass out of the vascular bed in tumor tissue during the time period between administration and imaging (e.g. particle sizes of 0.003 to 0.8 μm) and the particle size of a second population being such that they are retained in the vascular bed in tumor tissue during the time period between administration and imaging (e.g. particle sizes of 2 to 15 μm), and using said imaging modality detecting areas of the body where the biodistribution and clearance of the populations differ whereby to identify tumor tissue.

The labelling materials are preferably chromophores having different characteristic frequencies or radiolabels emitting different kinds or energies of radiation.

Viewed from a further aspect the invention also provides a diagnostic composition comprising at least two populations of physiologically tolerable particles, each said population being labelled with a material

detectable in the same diagnostic imaging modality, the mean particle size being different for each population and the labelling material being different for each population and interdistinguishable in said imaging modality, the particle size for one population being such that said particles pass out of the vascular bed in tumor tissue during the time period between administration and imaging and the particle size of a second population being such that they are retained in the vascular bed in tumor tissue during the time period between administration and imaging, optionally together with at least one pharmaceutically tolerable carrier or excipient.

Viewed from a still further aspect the invention provides the use of at least two populations of physiologically tolerable particles, each said population being labelled with a material detectable in the same diagnostic imaging modality, the mean particle size being different for each population and the labelling material being different for each population and interdistinguishable in said imaging modality, the particle size for one population being such that said particles pass out of the vascular bed in tumor tissue and the particle size of a second population being such that they are retained in the vascular bed in tumor tissue, for the manufacture of a diagnostic composition for use in a method of tumor location.

Either or both of the vesicles of different size may if desired also contain a cytotoxic therapeutic agent, e.g. cisplatin, carboplatin or paclitaxel. In this way vesicles detected at a tumor or other target site may be disrupted, e.g. by heating caused for example by photoirradiation, to release the cytotoxic agent. The imaging method allows for confirmation that the vesicles have reached the target site before the cytotoxic

therapeutic agent is released.

Similarly, the vesicles may contain or have bound thereto a contrast agent effective in another imaging modality, e.g. X-ray (CT), magnetic resonance (mr), ultrasound, nuclear imaging (i.e. scintigraphy, PET, SPECT, etc), magnetotomography, etc. Thus for example the vesicles may contain a gas or gas mixture and so also function as ultrasound contrast agents. By "gas" in this context is meant a material or mixture which is gaseous at the body temperature of the subject, which for humans is 37°C, e.g. oxygen, nitrogen, or a fluoroalkane, especially a perfluoroalkane containing up to 7 carbons, such as perfluoropentane, perfluorobutane or perfluorohexane (see, for example, WO92/22247).

Similarly, the vesicle may contain in its core an iodinated X-ray contrast agent (e.g. a soluble triiodophenyl compound, preferably a non-ionic compound such as iohexol, iopentol, iodixanol or iopamidol). (See, for example, WO95/26205.) Such vesicles will be suitable for contrast enhancement in both X-ray and light imaging modalities. In this instance it is especially preferred that the vesicles be dispersed in an aqueous solution of the X-ray contrast agent.

For dual functioning as light imaging and mr contrast agents, the vesicles may conveniently contain in their core a physiologically tolerable paramagnetic or superparamagnetic material, e.g. a transition metal or lanthanide chelate, especially a macromolecular chelate (e.g. Gd HPD03A), or superparamagnetic particles of a magnetic metal oxide, preferably an iron oxide. Such superparamagnetic-particle containing vesicles can function as contrast agents for both mr and magnetotomography when the vesicle membrane includes components (e.g. crown ethers) that permit water

transfer across the membrane. Alternatively, a paramagnetic persistent free radical may be used, as these will function both as radical scavengers and as mr contrast agents when the vesicle membrane includes components (e.g. crown ethers) that permit water transfer across the membrane. Alternatively, the vesicles may have polymeric or non-polymeric metal complexes capable of acting as mr contrast agents attached to their surfaces.

The vesicle compositions of the invention may be solids or dry powders or concentrated suspensions suitable for dilution prior to administration. Alternatively, they may be in ready-to-use form, i.e. in the form of suspensions or dispersions in a physiologically tolerable aqueous medium, e.g. physiological saline, water for injections, Ringers solution, etc. The compositions may contain conventional pharmaceutical excipients, such as for example pH adjusting agents, viscosity modifiers and freeze-drying aids, as well as soluble contrast agents (such as iohexol or gadodiamide), etc.

The chromophore in the compositions of the invention is preferably present at a content of 0.005 to 1.0 mmol/g membrane forming material and the compositions are preferably administered in a dose of 0.0001 to 0.01 mmol chromophore/kg bodyweight. Where other contrast agents are present, these are conveniently in concentrations/ dosages conventional for their particular imaging modality. Likewise, any cytotoxic agent present may be at or slightly below a conventional dosage level. Administration is preferably parenteral, e.g. by injection or infusion into the vasculature, intramuscularly or subcutaneously; however, administration by inhalation (e.g. of an aerosol spray) is also contemplated.

The light imaging procedure used with the contrast agents of the invention may be any conventional light imaging procedure - e.g. transmittance or reflectance imaging with incident laser light, and detection of the quasiballistic component of the light from the target zone, or photoacoustic (optoacoustic) imaging or acoustooptic imaging or confocal scanning laser microscopy (CSLM) or optical coherence tomography (OCT). The agents of the invention moreover may be used as contrast agents during surgical procedures so as to facilitate tumor margin delineation. In this way removal of healthy tissue and the risk of non-removal of tumorous tissue may both be minimized. In intra-operative use, the contrast agents may facilitate identification of the margins of surgical planes, i.e. allowing the surgeon to pass his instrument (e.g. a scalpel) between different tissue groups rather than through them. Thus viewed from a further aspect the invention provides a method of delineating a boundary to surgical intervention in a human or non-human animal body, said method involving administering to said body a light imaging contrast agent which distributes preferentially to one side of said boundary, said contrast agent being a composition comprising physiologically tolerable vesicles comprising (i) a vesicle membrane forming material, (ii) a chromophore material having a light absorption maximum in the range 300 to 1300 nm, and (iii) a scavenger, optionally together with at least one pharmaceutically acceptable carrier or excipient.

The procedure may also involve the use of focused ultrasound to modulate the light passing through the body location on which the ultrasound is focused and thus to facilitate separation of the light from that location from background light. Laser light may be directed at and light may be detected from an external

body surface, or alternatively an internal surface may be accessible by endoscope placement, e.g. through an existing or surgically created aperture in the body.

Viewed from a further aspect the invention thus provides a method of generating an image of a human or non-human animal (preferably a vascularized animal such as a mammal, bird or reptile) body which involves administering thereto a contrast effective amount of a light imaging contrast agent and using a light imaging modality generating an image of at least part of said body to which said agent distributes, the method being characterized by administering as said contrast agent a composition comprising physiologically tolerable vesicles which comprise (i) a vesicle membrane forming material, (ii) at least one chromophore material having a light absorption maximum in the range 300 to 1300 nm, and (iii) a scavenger, optionally together with at least one pharmaceutically acceptable carrier or excipient.

Viewed from a further aspect the invention provides the use of a physiologically tolerable scavenger, chromophore material and membrane forming material for the manufacture of a composition comprising physiologically tolerable vesicles which comprise (i) a vesicle membrane forming material, (ii) at least one chromophore material having a light absorption maximum in the range 300 to 1300 nm, and (iii) a scavenger, optionally together with at least one pharmaceutically acceptable carrier or excipient, for use in a method of diagnosis or treatment involving administration of said composition to a human or animal body and optionally the generation of an image using a light imaging modality of at least part of said body to which said vesicles distribute.

In one embodiment of the invention, a combination of a

micelle solubilized and water soluble form of a near infrared dye may be used for medical imaging of tumors, sites of inflammation, and other leaky vascular lesions. Thus, a dye (e.g. one of formula I) may be chemically modified such that an alkylphenyl group or other lipophilic moiety is attached to the chromophore without loss of the optical properties and further modified by the attachment of one or more high molecular weight polyethylene glycol (PEG) group (MW > 20 000) also without loss of the optical properties of the dye. An analogous dye may be prepared without PEG modification. If both dyes are injected into an artery of a rabbit with an implanted VX2 tumor in the thigh muscle, the low molecular weight analog may circulate for a significant time while also passively accumulating within the tumor itself as a result of its affinity for blood resident proteins and lipoproteins such as albumin, while the PEG modified dye may also circulate for a prolonged time period being both soluble and of high molecular weight. The different distributions of the dyes may be used to identify tumor sites, sites of inflammation or other leaky vasculature.

In another embodiment, chemical modification of a near infrared dye may be used for medical imaging of tumors, sites of inflammation, and other leaky vascular lesions as a result of absorption onto normally circulating proteins and lipoproteins. Thus, a dye may be chemically modified such that an alkylphenyl group or other lipophilic moiety is attached to the chromophore without loss of the optical properties. The relative hydrophilicity of this molecule may be such that it is partitioned into the membrane of a vesicular carrier but also retains a significant concentration in the aqueous phase, both inside the vesicle in the aqueous pool and in the continuous aqueous phase outside the vesicle. That is to say that the partitioning is a dynamic event

governed by a thermodynamic distribution coefficient which is near 1 and can be adjusted to favour either the aqueous phase or the membrane phase by adjusting the nature of the alkylphenyl group or other lipophilic moiety attached to the molecule. Upon injection, the chromophore present in the aqueous phase rapidly equilibrates with the normally resident proteins and lipoproteins in the blood to afford a carrier which passively accumulates within tissues that demonstrate leaky vascular beds such as tumors, sites of inflammation, and oedema. The material in the membrane phase and in the aqueous pool within the liposome will circulate with the liposome carrier which if sufficiently large, will not demonstrate accumulation within leaky vascular beds.

The inclusion of a scavenger material in the chromophore-containing compositions of the invention has the added advantage of prolonging shelf life by reducing photodecomposition during storage.

According to a further aspect, the invention provides a method of increasing the stability of a physiologically tolerable vesicular composition comprising a vesicle membrane forming material and a chromophore having a light absorption maximum in the range 300 to 1300 nm, said method comprising the step of incorporating into said composition a physiologically tolerable scavenger.

All publications and patent applications referred to herein are hereby incorporated by reference.

The invention will now be described further with reference to the following non-limiting Examples.

Example 1

Preparation of liposomes

Liposomes of average diameter 300 to 600 nm are prepared analogously to the method described in "Thin film hydration method" by A. D. Bangham *et al*. "Methods in Membrane Biology" (E. D. Korn, ed.), Plenum Press, NY, pp. 1-68 (1974). The maximum batch size produced by the process is 2.0 l. Hydrogenated phosphatidylcholine (10 g H-PC) and hydrogenated phosphatidylserine (1 g H-PS) are dissolved in chloroform/methanol/water (4:1:0.025 volume ratios) by shaking in a water bath at 70°C. The solvents are removed by rotary evaporation until a dry mixture of the phospholipids appears. The phospholipid mixture is added to an aqueous, 5% by weight mannitol solution in an amount sufficient to give a final suspension containing 10% by weight of the liposomal material, and the mixture is homogenized with a monomixer (6000 rpm for 10 minutes at a temperature of 65-70°C). The liposomes formed are extruded once through three polycarbonate filters (0.5 μm). Of the liposome suspension, 5.0 ml volumes are filled into 20 ml glass bottles, which are closed with grey rubber stoppers and sealed with aluminium caps. The liposomes are sterilized by autoclaving (at 121°C for 20 minutes).

Example 2

Preparation of liposomes containing dyes and stabilizer

A stabilized liposome containing a cyanine dye is made by adding 10 mg of a dye of formula I (wherein X=S, n=1, and R=$C_{18}H_{37}$) with a 5-fold molar excess of β-carotene to 10 ml of the mixture of Example 1 under heating at 60 to 70°C. The liposome mixture is further processed in one

of two ways:

(a) The first procedure uses a Microfluidizer®, which reduces the vesicle size with multiple passes through the processing chamber. As the phospholipid mixture heats up, the vesicles become smaller. At the desired size (e.g. 0.5 μm), the processing is stopped, and the mixture is sterile filtered into vials of the appropriate size to be autoclaved for sterilization.

(b) The second procedure involves extrusion of the dye, stabilizer and phospholipid mixture at about 80°C(±20°C), with a heated device (e.g. a water jacketed extruder) through a filter chamber containing polycarbonate filter(s) of the desired pore size (e.g. 0.5 μm). The collected filtrate can be autoclaved for sterilization. The sterile filtration is done in order to remove any particulates larger than 0.2 μm that may have resulted from the metal probe or the homogenizer.

Example 3

Preparation of liposomes containing dyes and stabilizer

The dye and stabilizing agent used in Example 2 are added to the phospholipid mixture of Example 1 in an aqueous medium (such as phosphate buffered saline or sterile water for injection) prior to processing with an ultrasonic probe, or tissue homogenizer to form the liposomal system. Once the liposomes containing dye and stabilizer have been formed, they are processed as in Example 2(a).

Example 4

Preparation of liposomes containing dyes and stabilizer

The procedure of Example 2 is repeated using Trolox® esters (esters of 6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid) in place of β-carotene.

Example 5

Kinetic study of the rate of degradation of 3,3'-diethylthiacarbocyanine iodide (DCI) in water, in liposomes, and in liposomes with added butylated hydroxytoluene

An assessment of the stability of 3,3'-diethyl-thiacarbocyanine iodide (DCI) in water, liposomes, and liposomes with added radical scavenger (butylated hydroxytoluene (BHT)) was carried out by measuring the absorbance at 769 nm over time using a spectrophotometer. DCI was purchased from Aldrich Chemical Co. (St. Louis, MO). Liposomes were prepared by extrusion of an aqueous stock solution of saturated phospholipids through 1 micron pore size filters under pressure. Liposomes prepared in this manner were characterized with an average particle size of 550 nm. Due to significant scattering from these concentrated liposomes, they were diluted 100x before use in the experiment described herein. BHT was added by placing a small amount of BHT into the diluted solution of liposomes to establish a saturated solution (with respect to BHT). The sample was prepared by adding a small amount of solid dye to methanol as a stock solution. The sample for analysis was prepared by adding approximately 20 microlitres of the stock solution in methanol to 10 ml of the solution of

interest (i.e. water, BHT, liposomes, or liposomes + BHT). 1 ml of the resulting mixture was placed into a quartz cuvette and the absorbance was monitored at 779 nm over time.

As shown in Figure 1, DCI in water alone degrades significantly over the 15 hour time course of the experiment. Addition of BHT slows the degradation somewhat, but not completely. In contrast, the absorbance in the presence of liposomes is fairly constant over the time course of the experiment. However, the degradation of the dye is slowest in the presence of both liposomes and BHT, indicating a synergistic effect of liposomes and scavengers.

Claims

1. A composition comprising physiologically tolerable vesicles which comprise:

(i) a vesicle membrane forming material;

(ii) at least one chromophore material having a light absorption maximum in the range 300 to 1300 nm; and

(iii) a scavenger;

optionally together with at least one pharmaceutically acceptable carrier or excipient.

2. A composition as claimed in claim 1 wherein the scavenger is a radical scavenger.

3. A composition as claimed in claim 1 wherein the scavenger is an antioxidant, a singlet oxygen quencher, or a chain transfer agent.

4. A composition as claimed in claim 1 wherein the scavenger is a singlet oxygen quencher.

5. A composition as claimed in claim 1 comprising two or more chromophore materials having a light absorption maximum in the range 300 to 1300 nm.

6. A composition as claimed in claim 5 wherein the chromophore materials are selected from phthalocyanines, naphthalocyanines, cyanines, squaryliums, pyryliums, thiapyryliums, anthraquinones, naphthaloquinones, triphenylmethanes, azo dyes, merocyanines, perylenes, and metal-complexed dyes.

7. A method of generating an image of a human or non-human animal body which involves administering thereto a contrast effective amount of a light imaging contrast agent and using a light imaging modality generating an

image of at least part of said body to which said agent distributes, the method being characterized by administering as said contrast agent a composition comprising physiologically tolerable vesicles which comprise:

(i) a vesicle membrane forming material;

(ii) at least one chromophore material having a light absorption maximum in the range 300 to 1300 nm; and

(iii) a scavenger;

optionally together with at least one pharmaceutically acceptable carrier or excipient.

8. A method of delineating a boundary to surgical intervention in a human or non-human animal body, said method involving administering to said body a light imaging contrast agent which distributes preferentially to one side of said boundary, said contrast agent being a composition comprising physiologically tolerable vesicles which comprise:

(i) a vesicle membrane forming material;

(ii) at least one chromophore material having a light absorption maximum in the range 300 to 1300 nm; and

(iii) a scavenger;

optionally together with at least one pharmaceutically acceptable carrier or excipient.

9. A method of increasing the stability of a physiologically tolerable vesicular composition comprising a vesicle membrane forming material and at least one chromophore material having a light absorption maximum in the range 300 to 1300 nm, said method comprising the step of incorporating into said composition a physiologically tolerable scavenger.

10. A method as claimed in any one of claims 7 to 9

wherein the scavenger is a radical scavenger.

11. A method as claimed in any one of claims 7 to 9 wherein the scavenger is an antioxidant, a singlet oxygen quencher, or a chain transfer agent.

12. A method as claimed in any one of claims 7 to 9 comprising two or more chromophore materials having a light absorption maximum in the range 300 to 1300 nm.

13. A method as claimed in claim 12 wherein the chromophore materials are selected from phthalocyanines, naphthalocyanines, cyanines, squaryliums, pyryliums, thiapyryliums, anthraquinones, naphthaloquinones, triphenylmethanes, azo dyes, merocyanines, perylenes, and metal-complexed dyes.

14. The use of a physiologically tolerable scavenger, chromophore material and membrane forming material for the manufacture of a composition comprising physiologically tolerable vesicles which comprise:

(i) a vesicle membrane forming material;

(ii) at least one chromophore material having a light absorption maximum in the range 300 to 1300 nm; and

(iii) a scavenger;

optionally together with at least one pharmaceutically acceptable carrier or excipient, for use in a method of diagnosis or treatment involving administration of said composition to a human or animal body and optionally the generation using a light imaging modality of an image of at least part of said body to which said vesicles distribute.

15. A method of detecting tumors in a human or animal body, said method comprising parenterally administering to said body, sequentially, separately or preferably

simultaneously, at least two populations of physiologically tolerable particles, each said population being labelled with a material detectable in the same diagnostic imaging modality, the mean particle size being different for each population and the labelling material being different for each population and interdistinguishable in said imaging modality, the particle size for one population being such that said particles pass out of the vascular bed in tumor tissue during the time period between administration and imaging and the particle size of a second population being such that they are retained in the vascular bed in tumor tissue during the time period between administration and imaging, and using said imaging modality detecting areas of the body where the biodistribution and clearance of the populations differ whereby to identify tumor tissue.

16. A method as claimed in claim 15 wherein said modality is selected from light imaging, magnetic resonance imaging, X-ray imaging, nuclear imaging, ultrasound imaging, photoacoustic (optoacoustic) imaging, acoustooptic imaging, confocal scanning laser microscopy (CSLM) or optical coherence tomography (OCT).

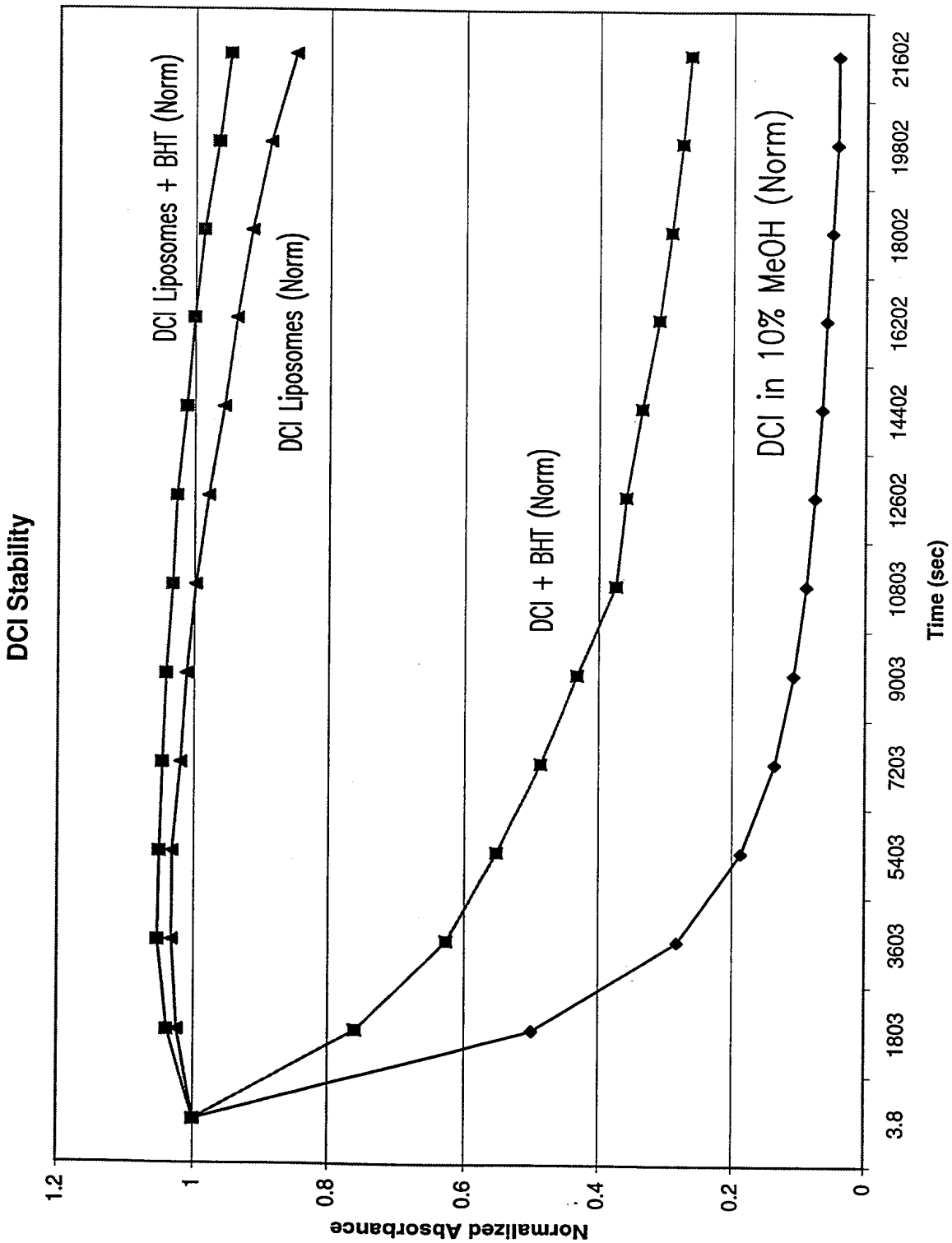
17. A diagnostic composition comprising at least two populations of physiologically tolerable particles, each said population being labelled with a material detectable in the same diagnostic imaging modality, the mean particle size being different for each population and the labelling material being different for each population and interdistinguishable in said imaging modality, the particle size for one population being such that said particles pass out of the vascular bed in tumor tissue and the particle size of a second population being such that they are retained in the vascular bed in tumor tissue, optionally together with

at least one pharmaceutically tolerable carrier or excipient.

18. The use of at least two populations of physiologically tolerable particles, each said population being labelled with a material detectable in the same diagnostic imaging modality, the mean particle size being different for each population and the labelling material being different for each population and interdistinguishable in said imaging modality, the particle size for one population being such that said particles pass out of the vascular bed in tumor tissue and the particle size of a second population being such that they are retained in the vascular bed in tumor tissue, for the manufacture of a diagnostic composition for use in a method of tumor location.

19. Use as claimed in claim 18 wherein said particles are contrast enhancing in at least one imaging modality selected from light imaging, magnetic resonance imaging, X-ray imaging, nuclear imaging, ultrasound imaging, photoacoustic (optoacoustic) imaging, acoustooptic imaging, confocal scanning laser microscopy (CSLM) or optical coherence tomography (OCT).

Fig. 1


DCI Stability
DCI Liposomes + BHT (Norm)
DCI Liposomes (Norm)
DCI + BHT (Norm)
DCI in 10% MeOH (Norm)
Normalized Absorbance
1.2
1
0.8
0.6
0.4
0.2
0
Time (sec)
3.8
1803
3603
5403
7203
9003
10803
12602
14402
16202
18002
19802
21602